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New Insights into Cheese Microstructure

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Abstract

Microscopy is often used to assist the development of cheese products, but manufacturers can benefit from a much broader application of these techniques to assess structure formation during processing and structural changes during storage. Microscopy can be used to benchmark processes, optimize process variables, and identify critical control points for process control. Microscopy can also assist the reverse engineering of desired product properties and help troubleshoot production problems to improve cheese quality. This approach can be extended using quantitative analysis, which enables further comparisons between structural features and functional measures used within industry, such as cheese meltability, shreddability, and stretchability, potentially allowing prediction and control of these properties. This review covers advances in the analysis of cheese microstructure, including new techniques, and outlines how these can be applied to understand and improve cheese manufacture.

1. INTRODUCTION

Microscopy techniques provide an opportunity to understand the development of cheese structure at the micron scale. Much of the work in this field has focused on final product structure, as this determines the textural and functional properties of the cheese, affecting both product performance and customer sensory perception (Lamichhane et al. 2018). Yet the potential for microscopy to add value in dairy manufacturing is much broader, as this technique can also inform manufacturers about the process of manufacturing.

Microscopy can reveal how the microstructure forms within intermediate samples, owing to the action of processing steps and as a function of process variables such as temperature, pH, and shear. Sample structure can be assessed throughout the manufacturing process, beginning with the quality of the raw ingredients, followed by the development of key structural traits during manufacture, the characteristics of final products, and any subsequent changes or degradation in structure during storage and in the supply chain. These insights allow manufacturers to:

- Benchmark and design new processes;
- Optimize existing processes;
- Reverse engineer particular properties; and
- Troubleshoot production problems.

In this way, microscopy is applied as a tool to complement process design and optimization.

This review aims to provide a broad overview of the application of microscopy in the optimization of dairy products and manufacturing processes. It first defines cheese microstructure, provides an overview of how microstructure is generated, and describes the importance of these structural features (Section 2). The instruments used to study cheese microstructure, including new techniques, are discussed together with the additional benefits of quantitative image analysis (Section 3). The review then explores how microstructure analysis can be applied to assist manufacturers and food engineers. This includes the application of microscopy to assess the structure of the raw materials used to make cheese products (Sections 4.1–4.2) and as a tool to complement process optimization and control (Sections 4.3–4.6). The potential to apply these methods to other products, including nonbovine milk and new plant-based products, is also outlined (Section 4.5). Finally, the future direction of microscopy in cheese processing is anticipated.

2. WHAT IS CHEESE MICROSTRUCTURE, HOW IS IT GENERATED, AND WHY IS IT IMPORTANT?

Microstructure is one of the three length scales describing structures found within cheese. These include (Foegeding et al. 2017):

1. The molecular or “nano-scale.”
2. The microscopic or “meso-scale.”
3. The visual or “macroscale.”

The microstructure of a cheese describes the arrangement of the cheese components, which are mainly derived from milk, including protein, fat, carbohydrates, and the serum that contains minerals. The raw ingredients present within milk can be likened to the building blocks of a complex architectural design that can be assembled into a structure with desirable sensory and textural properties. Although the chemical composition of these ingredients often plays a significant role in the stability and nutritional properties of the cheese, it is the assembly of the macromolecules that gives rise to the microstructure (Aguilera et al. 2000), determining the product properties and quality.

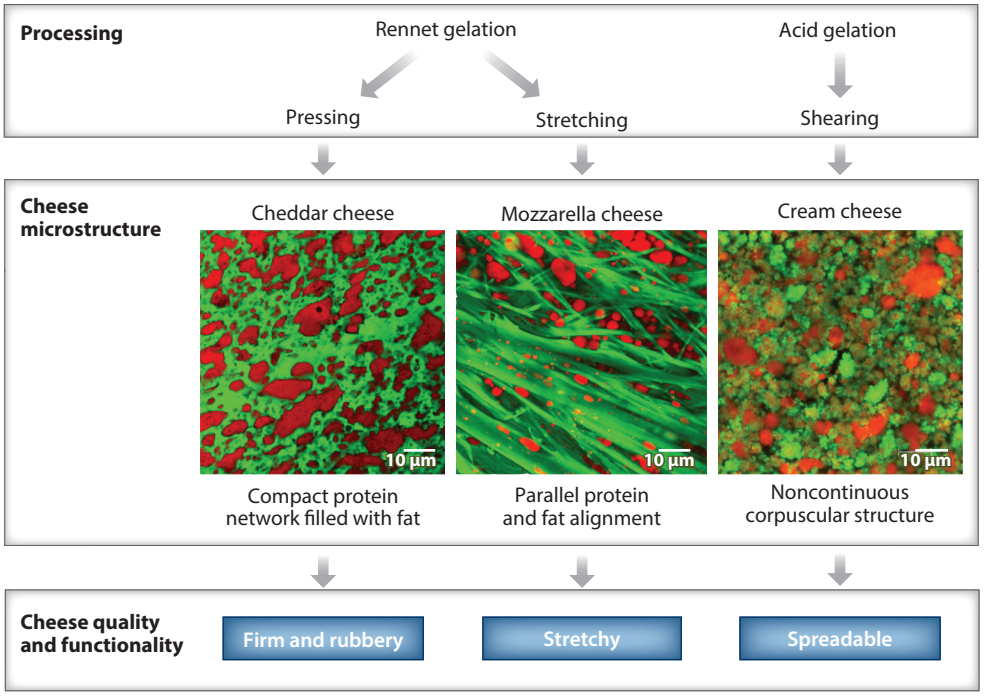


Figure 1

Processing determines cheese microstructure, which impacts cheese quality and functionality. During processing, protein is first altered and then assembled by either rennet or acid gelation. This is followed by further deformation (pressing and stretching) or shear, which alters the arrangement of fat and protein, resulting in different microstructures for Cheddar cheese, high-moisture Mozzarella cheese, and cream cheese. Fat appears red and protein appears green in these images. The scale bars are 10 µm in length. Image of Cheddar cheese and Mozzarella cheese used with permission from Ong et al. (2017) and Nguyen et al. (2017b). Image of cream cheese is an unpublished image from L. Ong and S.L. Gras.

The manufacturing process determines product microstructure, as it affects molecular assembly and the final arrangement of building blocks (as shown in **Figure 1**). The different processing steps lead to the characteristic microstructures of Cheddar cheese, high-moisture Mozzarella, cream cheese, or other products. In **Figure 1**, the distinct arrangement of protein and fat in these three products is visible due to the fluorescence staining. The Cheddar cheese consists of a compact protein network, filled with fat. The high-moisture Mozzarella contains an anisotropic structure with parallel alignment of protein and fat, and the cream cheese contains a noncontinuous network (i.e., there are spaces between discrete protein-filled areas) described as a corpuscular structure (i.e., small particles suspended in a fluid). Each of the three cheese microstructures gives rise to different characteristic functional properties (**Figure 1**), giving the consumer a range of functional and sensory experiences.

The process of manufacturing and its influence on product microstructure can be divided into three major steps (van der Sman 2012), providing a useful framework for understanding structure development. These steps are:

1. The modification of the native structure of the raw materials during manufacture.
2. The creation of new structures during manufacture.
3. The stabilization of created structures via arrest in a jammed state.

The modification of the native structure begins with initial milk processing, including the collection, pumping, and storage of milk. Most notable are steps such as high-pressure processing or homogenization that reduce the size of the native milk fat globule and lead to the formation of new clusters of fat and protein, providing the opportunity for many different product textures.

The creation of new microstructures during manufacturing often relies on the self-assembly of proteins. Milk proteins are typically altered by (Nicolai & Durand 2013):

1. Enzyme addition, such as rennet (rennet gelation).
2. pH alteration through the addition of microbial starter cultures or acidulants (acid gelation).
3. Protein denaturation and aggregation by heating (heat gelation).
4. The combination of two or three of these mechanisms.

Examples of processing steps involving the alteration and assembly of proteins include the rennet and acid gelation shown in **Figure 1**. Protein and fat are initially assembled into a coagulum or gel, which is then further processed by deformation (e.g., via pressing and stretching) or shearing (**Figure 1**). The choice of steps gives rise to a range of unique microstructures. The structure is then typically further arrested in a jammed state (e.g., via cooling) to produce stable cheese products that differ in final microstructure (**Figure 1**).

During storage, shelf-stable arrested cheese structures are prone to further structural alterations due to physical changes and biochemical reactions occurring within the cheese. These processes include the absorption of water in Mozzarella cheese, which occurs over the first two weeks of storage, as well as proteolytic, glycolytic, and lipolytic reactions that occur during the ripening of a range of cheeses (Khattab et al. 2019).

The microstructure of cheese is of particular importance, as it can be sensed by the human tongue (van der Sman 2012). Although the structures found at all length scales contribute to product properties and customer perception, it is the microscopic structures, i.e., the features that are micrometers in length, that have the largest potential to impact product quality (Aguilera 2005). Food components at this length scale can also participate in transport properties (e.g., mass transfer by molecular diffusion), affecting the quality and stability of the product during storage, and many key phenomena (e.g., gelation, homogenization, crystallization) occur at the microstructural scale (Aguilera 2005). The correlations between food microstructure, sensory, nutritional, chemical, microbiological stability, texture, and transport properties have been reviewed by Aguilera (2005), Foegeding et al. (2017), Everett & Auty (2017), and Lamichhane et al. (2018). These reviews highlight how the design of a particular food or cheese microstructure can impart specific product qualities and functionalities.

Cheese microstructure also has the potential to influence digestion and health. The microstructure is initially broken down during chewing and mastication in the mouth. In the digestive tract the components are then degraded, allowing adsorption. Several studies have investigated the link between cheese microstructure and its breakdown during in vitro digestion (Ayala-Bribiesca et al. 2016, Lamothe et al. 2012, Mulet-Cabero et al. 2017). These studies show that the physical characteristics of the cheese, including the texture and microstructure, influence degradation and the release of nutrients. Textural characteristics, such as cheese cohesiveness and elasticity, were associated with slower degradation, and denser protein matrixes restricted the access of lipase to cheese fat, lowering the release of fatty acids (Lamothe et al. 2012), illustrating the potential to tailor these properties.

The potential link between food microstructure and digestion provides further motivation to understand product microstructure, as recent advances have linked digestion with the human microbiome, immunity, and general health (Lloyd-Price et al. 2016, Malla et al. 2019, McBurney et al. 2019, Pflughoeft & Versalovic 2012). These studies highlight the need to better understand and

preserve native food microstructure or reliably control and produce desired microstructures. The controlled production of specific desired microstructures will assist food manufacturers, allowing them to target new product categories, make claims such as satiety, influence the gut microbiome, and target particular consumers or age groups (Li-Chan & Nakai 1989).

3. MICROSCOPY TECHNIQUES

3.1. Overview of Microscopy Techniques for the Characterization of Cheese Microstructure

There is a range of established and emerging techniques that can be used to assess cheese microstructure (see **Table 1**). Several common techniques, including light microscopy, confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM), have been reviewed in detail (Burdikova et al. 2015a, El-Bakry & Sheehan 2014, Everett & Auty 2017). Most techniques are used to characterize the components present in cheese, although fluorescence lifetime imaging microscopy (FLIM) has been applied to assess local pH, fluorescence recovery after photobleaching (FRAP) to study diffusion, and atomic force microscopy (AFM) to study local mechanical properties.

Super-resolution techniques allow images to be taken with a higher resolution beyond the diffraction-limited resolution of conventional light microscopy. These techniques are relatively new but are likely to find greater application because of their ability to provide greater structural detail. 3D structured illumination (SIM) super-resolution microscopy, for example, has been applied to analyze the subcellular structure of nonpathogenic spore-forming Clostridia in Grana-type cheeses (D'Incecco et al. 2018a). This technique overcomes the diffraction limit and improves the lateral resolution to 30–150 nm (compared to 200–300 nm in a standard fluorescence microscope) (Schermelleh et al. 2019) but has the disadvantage of requiring chemical fixation. Stimulated emission depletion (STED) can also generate high-resolution images of acid gel microstructure, including protein domains, interpore distances, and fractal dimensions (Glover et al. 2020). Other high-resolution techniques that allow single-molecule localization with nanometer precision include photoactivation localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM). PALM has been used to study particles within *Lactococcus lactis* bacterial cells (**Table 1**), but its broader potential has not yet been fully explored.

Microscopy has also been combined with spectroscopy to develop techniques that can provide additional information on molecular components, including Fourier transform infrared (FTIR) microspectroscopy, confocal Raman, coherent anti-Stokes Raman scattering (CARS), stimulated Raman scattering (SRS), Raman integrated scanning electron (RISE) microscopy, and AFM Raman (Dufour 2011, Lei & Sun 2019, Wellner 2013) (**Table 1**). FTIR and Raman microscopy avoid the need for fluorescence staining, and Raman spectroscopy is particularly useful for lipid-rich products, which cause significant scattering. CARS can further enhance weak Raman signals, improving image resolution and imaging speed. A useful example of Raman includes RISE microscopy, which has been used to chemically map structures observed during electron microscopy. In the study by Quigley et al. (2016), this technique was useful to detect the presence of lycopene in an area of pink discoloration on cheese (**Table 1**). The combination of AFM with infrared (AFM-IR), scanning near-field optical microscopy (AFM-SNOM), or tip-enhanced Raman scattering (AFM-TERS) enables chemical mapping of the structure of fat globules, cell membranes, or bacterial spores (**Table 1**) and has the potential to study surface structures and mechanical properties. Another complementary combination is the use of microscopy with rheological or textural analysis (Lamichhane et al. 2020), allowing the simultaneous collection of microstructural and mechanical information (**Table 1**).

Table 1 Microscopy techniques that can characterize the microstructure of cheese

Microscopic technique	Typical samples	Purpose of analysis	Reference(s)
Commonly used techniques			
LM, PLM	Processed cheese, soft smear-ripened cheese, Mozzarella cheese, Oaxaca cheese	Characterization of starch, including the effect of processing on starch distribution Identification of surface crystals Assessment of water after freezing and thawing	Alinovi et al. 2020a, de Los Ángeles Colín-Cruz et al. 2012, Lenze et al. 2019, Polowsky et al. 2018a, Talbot-Walsh et al. 2019
CLSM	A variety of cheeses	Analysis of cheese matrix during processing and storage To establish structure–function relationships	Banville et al. 2016, Ma et al. 2013, Macdougall et al. 2019, Ong et al. 2020a, Yang et al. 2016
SHG	Cheddar cheese	Analysis of the distribution of calcium phosphate crystals	Burdikova et al. 2015a
FLIM	Natural cheese, Swiss-type cheese, milk acid gels	Analysis of pH heterogeneity within the cheese matrix Localization of phospholipids in the cheese matrix Analysis of moisture loss in acid milk gels	Burdikova et al. 2015b, Glover et al. 2020
FRAP	A variety of cheeses, milk protein gels	To understand transport phenomena and diffusion coefficients of dextran, proteins, peptides, and enzymes such as pepsin and other small or macromolecules in a food matrix	Chai et al. 2018; Chapeau et al. 2016; Floury et al. 2012; Guo et al. 2017; Silva et al. 2013, 2015; Thévenot et al. 2017
SEM, cryo-SEM, TEM, cryo-TEM	A variety of cheeses	Characterization of the microstructure of casein micelles and fat globules Analysis of the cheese matrix during processing and storage Characterization of mineral crystal complexes Characterization of exopolysaccharide-producing bacteria in cheese	Dabour et al. 2006, D'Incecco et al. 2016, Kuo & Gunasekaran 2009, Lazzaro et al. 2020, Ong et al. 2011, Vollmer et al. 2019
AFM	Milk relevant for cheese-making	Quantification of the nanomechanical properties of the MFGM during the processing of Cheddar cheese Measurement of the adhesive force between fat globules and casein micelles	Balasuriya et al. 2012, Obeid et al. 2019
Super-resolution techniques			
Structured illumination microscopy	Hard cheese	Visualization of the subcellular structure within *Clostridium* spores	D'Incecco et al. 2018b
Stimulated emission depletion	Acid milk gels	Quantification of the length of protein domains, interpore distances, and fractal dimension of acid gels	Glover et al. 2020
Photoactivation localization microscopy	*Lactococcus lactis* bacterial cells	Single-particle tracking of proteins within *Lactococcus lactis* reveals differences in the internal dynamics between cells	Beljouw et al. 2019

(*Continued*)

Table 1 (*Continued*)

Microscopic technique	Typical samples	Purpose of analysis	Reference(s)
Integrated systems that combine imaging techniques with spectroscopy or tensile deformation			
FTIR spectroscopy	Mozzarella cheese	Characterization of fat and protein distribution in cheese	Pax et al. 2019
Confocal Raman	Processed cheese	Characterization of additives, e.g., emulsifiers, acid, and starch, in processed cheese and their distribution	Smith et al. 2017
CARS, SRS	Cheese	Assessment of hydration dynamics in cheese as a function of time Characterization of biomolecules such as proteins, lipids, and water	Glover et al. 2020, Roeffaers et al. 2010
RISE microscopy	Ripened cheese	Raman chemical mapping of a region of defective pink discoloration on an electron microscope image	Quigley et al. 2016
AFM-IR, AFM-SNOM, AFM-TERS	Bacterial cells, *Bacillus subtilis* spores, starch granules	Localization of triglyceride vesicles in bacteria (AFM-IR) Assessment of protein–surfactant interactions and the action of glycoamylases on starch granules (AFM-SNOM) Chemical fingerprinting of the surface of *Bacillus subtilis* spores (AFM-TERS)	Barlow et al. 2016, Liu & Yang 2019, Morris 2004, Rusciano et al. 2014
Tensile deformation	Semihard cheese	Dynamic in situ imaging of cheese to understand fracture behavior	Lamichhane et al. 2020
Imaging techniques based on magnetic resonance and X-ray			
MRI	Semihard cheese, Emmentaler, Grana Padano	Estimation of the number, volume, and spatial distribution of eyes in semihard cheese during ripening Characterization of curd granules and curd junctions of Emmentaler cheese with 3D magnetic resonance microimaging Assessment of Grana Padano ripening by investigating the transverse relaxation time (T_2)	Huc et al. 2014, Mahdjoub et al. 2003, Mulas et al. 2016, Musse et al. 2014
X-ray and X-ray computed tomography	Semihard cheese, hard cheese, cream cheese, Gouda-type cheese, Swiss-type cheese	Quantification of the number, volume, and size distribution of eyes in cheese Characterization of the microstructure and number of fat droplets in cream cheese	Bisig et al. 2019; Guggisberg et al. 2013; Huc et al. 2014; Kraggerud et al. 2009; Laverse et al. 2011; Lee et al. 2012; Schuetz et al. 2013, 2016

Abbreviations: AFM, atomic force microscopy; CARS, coherent anti-Stokes Raman scattering; CLSM, confocal laser scanning microscopy; FLIM, fluorescence lifetime imaging microscopy; FRAP, fluorescence recovery after photobleaching; FTIR, Fourier transform infrared; IR, infrared; LM, light microscopy; MFGM, milk fat globule membrane; MRI, magnetic resonance imaging; PLM, polarized light microscopy; RISE, Raman integrated scanning electron; SEM, scanning electron microscopy; SHG, second harmonic generation; SNOM, scanning near-field optical microscopy; SRS, stimulated Raman scattering; TEM, transmission election microscopy; TERS, tip-enhanced Raman scattering.

Larger features, including macro structures such as cheese eyes, have been imaged using magnetic resonance or X-rays, such as magnetic resonance imaging (MRI) and X-ray-computed tomography (CT) (**Table 1**). X-ray CT has also been applied to image smaller features, such as fat droplets in cream cheese (Laverse et al. 2011). Other applications for X-ray-based imaging

techniques in the characterization of food microstructure are provided by Schoeman et al. (2016).

Techniques yet to be applied to study cheese microstructure include electronic preresonance conditions (Epr)-SRS, which provides greater sensitivity and vibrational contrast than a standard confocal Raman microscopy and provides an enhanced detection limit for small protein components (Wei et al. 2017).

3.2. Quantitative Image Analysis Techniques and Prediction of Functional Properties

Quantitative image analysis can be performed on images collected from a range of microscopy techniques (**Table 1**), providing additional complementary information that allows objective interpretation, which is difficult by eye (El-Bakry & Sheehan 2014, Fenoul et al. 2008). Useful data can be generated, such as the size distribution and shape of curd granules in hard and semihard Swiss cheeses (Ruegg & Moor 1987), the shape and size of fat globules in Cheddar cheese (Ding & Gunasekaran 1998), and the porosity, pore size, protein branching (vertices) (Ong et al. 2011, 2015, 2018), length of protein domains, interpore distances, and fractal dimensions for acid gels (Chapeau et al. 2016, Glover et al. 2020).

The typical workflow for quantitative image analysis is shown in **Figure 2**, together with images illustrating the process of segmentation and the generation of 3D-rendered surfaces of fat and protein obtained from 3D confocal images (**Figure 2*a***). The collection and analysis of

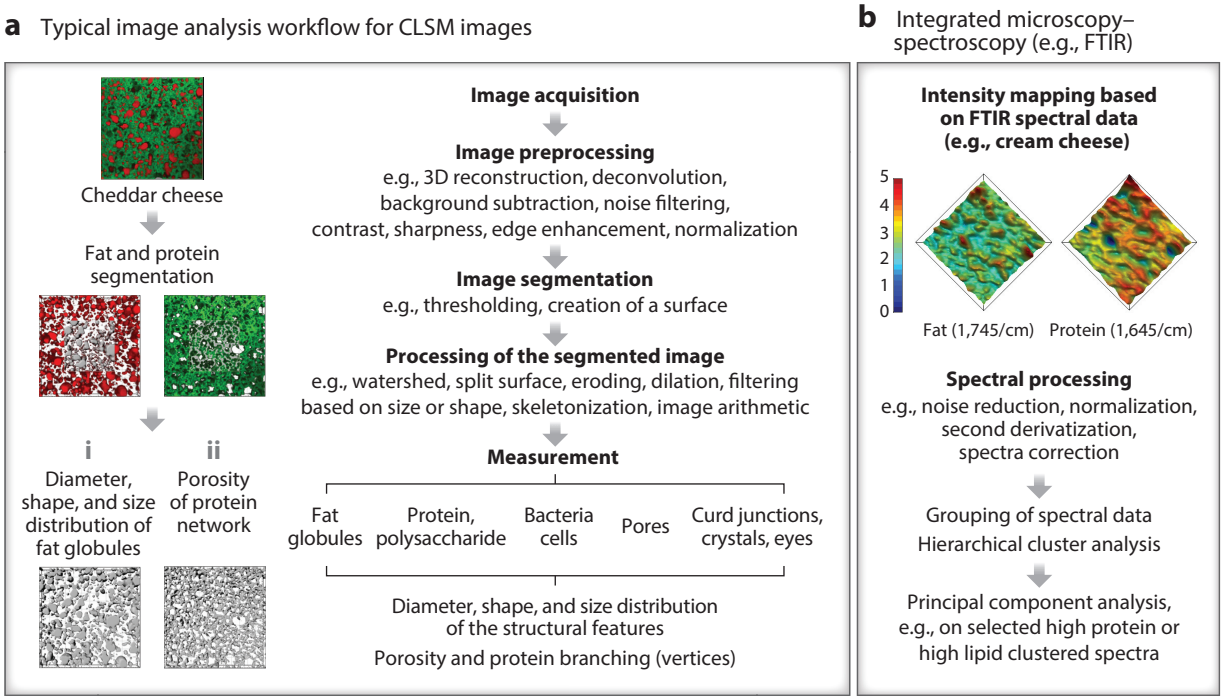


Figure 2

(*a*) Typical workflow applied for the quantitative analysis of images of cheese microstructure. Confocal laser scanning microscopy (CLSM) images of Cheddar cheese processed using Imaris image analysis software to quantify (*i*) the diameter, shape, and size distribution of fat globules and (*ii*) the porosity of the protein network. (*b*) Spectral processing and principal component analysis applied to process Fourier transform infrared (FTIR) spectral data of cream cheese samples. Images are from L. Ong and S.L. Gras.

3D information are essential for accurate interpretation of microstructural characteristics and avoid the bias arising from the interpretation of 2D images or the need to apply stereological approaches. For example, 2D sections taken at different heights through a fat globule give variable diameters (Everett & Olson 2003, Ong et al. 2011).

Large spectral data sets collected from techniques, such as Raman and FTIR, can also be analyzed using techniques such as hierarchical cluster analysis and principal component analysis, allowing differences between spectral patterns to be explored (**Figure 2*b***). These techniques reduce complexity, which assists analysis (Ong et al. 2020b, Pax et al. 2019).

Quantitative analysis of microscopy images and spectral data provides valuable information that can help establish structure–function relationships. Researchers have also utilized a variety of mathematical methods, including partial least squares regression (PLSR), artificial neural networks, and convolutional neural networks to obtain further insights (Lu et al. 2021, Vásquez et al. 2018). For example, hyperspectral imaging has been coupled with PLSR or artificial neural networks to predict the texture of Swiss cheese during ripening (Vásquez et al. 2018). This approach, using Big Data, will likely allow the optimization of processes and product functionality, although it requires rapid techniques, such as hyperspectral imaging that can be conducted at or in line for high throughput.

4. APPLYING MICROSCOPY TO ASSESS CHEESE MICROSTRUCTURE

Microscopy tools can be applied to follow and characterize the stages of cheese production. This includes the structure of the raw materials (Section 4.1), followed by the structural changes that begin during milk processing (Section 4.2) and continue to occur throughout cheese-making, affecting the structure of both protein (Section 4.2.1) and fat (Section 4.2.2). Microscopy can also be applied to complement process engineering during process benchmarking (Section 4.3), process optimization (Section 4.4), and reverse engineering (Section 4.5) as well as when troubleshooting production problems (Section 4.6).

4.1. Examining the Structure of the Building Blocks Used to Make Cheese and Dairy Products

Microscopy can be used to assess the native microstructure of the building blocks used to make cheese and as an initial indicator of raw material quality. These building blocks, shown in **Figure 3*a–c***, include casein micelles, whey protein, and fat globules. Fat is also covered in a surface-active membrane material, referred to as the milk fat globule membrane (MFGM). These ingredients range in length scale: The proteins and membrane materials are submicron in scale, whereas the fat droplets are largely micron in scale. Therefore, a range of microscopy techniques is required for visualization.

Casein micelles are polydisperse particles, ~100–200 nm in diameter, that can be visualized with high-resolution techniques such as TEM (**Figure 3*b***). They are composed of the casein proteins (α_{s1}, α_{s2}, β, and κ-casein) together with minerals that occur mainly in calcium phosphate nanoclusters (Lazzaro et al. 2020). Their structure is described by the early submicelle model (Walstra 1999), the dual-binding model (Horne 1998), and the nanocluster model (Holt et al. 1998).

Globular whey proteins, predominantly β-lactoglobulin and α-lactalbumin, represent ~20% of the total protein content in milk; these are harder to visualize by microscopy. They occur as nanometer-sized protein particles (Kharlamova et al. 2019) that when denatured can be seen using TEM. The denatured whey protein can also adhere to the surface of casein micelles (McKenna et al. 1999), although these are not easily distinguished from protruding κ-casein.

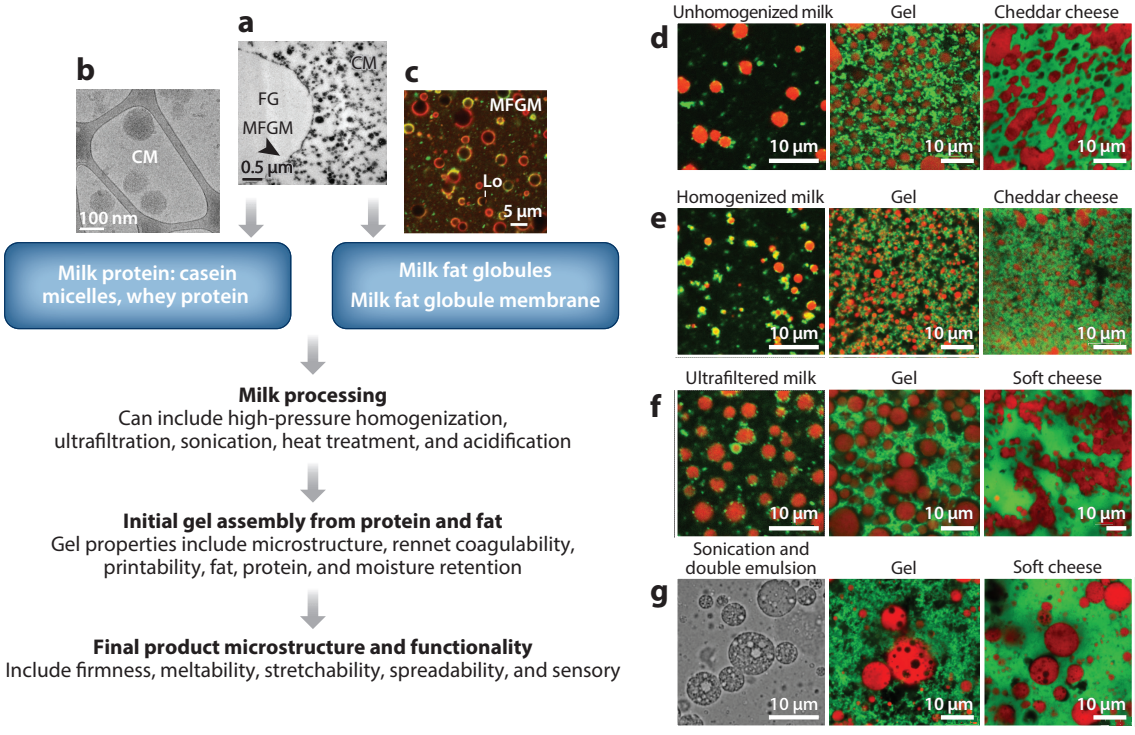


Figure 3

The application of microscopy to characterize the structure of raw materials and monitor the formation of intermediate and cheese microstructure during a manufacturing process. Left: The main building blocks in milk used to make cheese include casein micelles (CMs) (seen in panels *a* and *b*) and fat globules (FGs) (seen in panels *a* and *c*). The milk fat globule membrane (MFGM) is also seen in panels *a* and *c*. The structure of these components is altered during milk processing, which is followed by an initial assembly of proteins in a gel and then through subsequent processing forms the final product microstructure, influencing product functionality. Rhodamine dioleoylphosphatidyl ethanolamine–stained polar lipids appear red and lectin wheat germ agglutinin 488–stained glycoproteins and glycolipids appear green in panel *c*. Abbreviation: Lo, liquid-ordered domain consisting of sphingomyelin and cholesterol. Right: The different microstructures of the milk ingredients, initial gel assembly, and final cheese product arising from different milk processing steps (panels *d–g*). Cheese from (*d*) milk without homogenization, (*e*) homogenized milk, (*f*) milk concentrated by ultrafiltration, and (*g*) milk treated using sonication to form a double emulsion. The fat appears red and the protein appears green in panels *d–g*. Images in panels *d–f* adapted with permission from Ong et al. (2010). Images in panels *a*, *b*, *c*, and *g* are unpublished images from L. Ong and T.S.H. Leong.

Milk fat droplets range from 0.2 μm to 15 μm in diameter (Michalski et al. 2006, Ong et al. 2011) (**Figure 3*a*,*c*,*d***; unhomogenized milk) and can easily be seen using fluorescent staining and CLSM. Fat droplets are composed mainly of triacylglycerols and are covered with the surface-active MFGM (**Figure 3*c***). The components of this layer can also be visualized using fluorescent staining and CLSM. They include the polar lipids sphingomyelin and cholesterol, structured in rigid liquid-ordered (Lo) domains. A fluid matrix containing glycerophospholipids, proteins, glycoproteins, and glycolipids provides a surrounding liquid-disordered phase (Lopez et al. 2019). The native MFGM acts as an emulsifying agent that assists dispersion and prevents coalescence. It also interacts with the gastrointestinal tract (Lopez et al. 2019) and has several potential biological functions (Lopez et al. 2019, Nguyen et al. 2017a).

The degradation or association of these milk components are key factors affecting raw milk properties and quality. For example, casein micelles may aggregate during the storage of raw milk

due to bacterial or other proteolytic enzymes (Enright et al. 1999). Mechanical agitation during transport to a factory or during storage may also lead to lipolysis and fat globule coalescence (Deeth 2006). The persistence of fragile features, such as the native MFGM, in microscopic images can therefore be used as a marker to assess the extent to which the native structure is preserved during processing (Ong et al. 2010).

4.2. The Modification of Cheese Building Blocks During Milk Processing

Milk processing is typically the first step of cheese-making. It involves changes to the native microstructure induced by homogenization or other processing steps, where the concentration, structure, and properties of the native building blocks are intentionally altered (**Figure 3*a–c***). These changes can be characterized by microscopy, as shown by the different processed milk streams in **Figure 3*d–g***. Initial milk processing can improve ingredient properties during subsequent processing steps, for example, rennet coagulability during gel assembly. Milk processing can also be used to achieve a desired final product functionality, such as improved firmness, meltability, stretchability, spreadability, and sensory properties, as shown in **Figure 3**.

The modification of protein and fat components during manufacturing, including the initial milk, intermediate gel structure, and final product stages, as assessed by CLSM imaging, is shown in **Figure 3*d–g***. These steps are also explored in more detail in the following sections.

4.2.1. The modification of milk protein structure. The native protein structure may be altered by several initial milk processing steps (**Figure 3*a–c***). High-shear processing, such as homogenization, increases the association of milk protein with fat globules (**Figure 3*d,e***; milk). Shear within an ultrafiltration (UF) process can also lead to protein adsorption on fat droplets (**Figure 3*f***; ultrafiltered milk), although this is less extensive than in homogenized milk (**Figure 3*e***; homogenized milk).

The next stages during cheese-making involve the assembly of protein that occurs during gelation. The casein micelles fuse together to form 3D chains that are visible by CLSM (**Figure 3**; gel stage), with differences in the protein network leading to different cheese types (**Figure 3**; including Cheddar and soft cheeses). Process changes such as homogenization can alter the protein network in the final cheese product (**Figure 3*d,e***). Concentration by UF increases the total solids (typically two times the concentration of whole milk), lowering the mean distance between protein molecules and also altering protein aggregation (Ong et al. 2013). Larger regions of protein could be observed within the final cheese network from UF milk (**Figure 3*f***).

The native protein structure may also be altered by heat treatment and pH prior to the gelation process. These steps have a large impact on ingredient and gel properties. For example, altering the pH of the milk affects the solubilization of colloidal calcium phosphate, leading to changes in the size of casein micelles and rennet coagulation (Lazzaro et al. 2020). The combination of heat treatment and pH can also be used to alter the denaturation of whey proteins and their interaction with κ-casein, again affecting protein assembly. For example, increasing the pH of milk from 6.5 to 6.9–7.1 during heating results in the dissociation of some κ-casein from casein micelles, leading to the formation of complexes with denatured whey protein. The increased interaction between these complexes can increase aggregation, creating materials also suitable for 3D printing (Daffner et al. 2020).

4.2.2. The modification of milk fat globule structure. When native unhomogenized fat globules are used in cheese-making (**Figure 3*d***; unhomogenized milk), they appear as filler within the gel and cheese protein network (**Figure 3*d***; gel and Cheddar product). Homogenization reduces the size of these globules, decreasing the surface area covered by the native MFGM, leading to

protein adsorption (**Figure 3*e***; homogenized milk). The adsorbed protein forms a new recombined membrane that enables the fat globule to take part in the gelation process, contributing to gel and cheese network formation (**Figure 3*e***, gel and Cheddar cheese). The inclusion of fat globules within the protein network softens the network and reduces the ability of the protein to knit together during manufacture, resulting in cheese with higher moisture content that is less elastic and more crumbly (Ong et al. 2011). Homogenization can also increase the meltability and reduce free-fat formation in Mozzarella cheese (Rowney et al. 2003). The homogenization process results in smaller and more emulsified fat globules that interact with the protein matrix, reducing the likelihood of free-fat formation.

The process of UF, which involves shear forces, can also cause disruption to the native MFGM, leading to protein adsorption (**Figure 3*f***; ultrafiltered milk). These fat globules also appear as fillers within the protein network.

Sonication has also been used to modify the microstructure of the fat globules in cheese production. Analog cheese was successfully produced from skim milk and canola oil emulsified using ultrasound to form double emulsions (Leong et al. 2020) (**Figure 3*g***; double-emulsion milk). The small droplets of skim milk appeared within the fat phase in the rennet gel and were retained within the final cheese for 7 months at 4°C but the texture of the cheese was firmer and had a lower meltability than conventional Cheddar.

Other preprocessing methods include the size-based fractionation of fat droplets by microfiltration (MF). This process can alter the microstructure and texture of cheese (Logan et al. 2017, Michalski et al. 2006), with smaller native fat globules (~3 μm in diameter compared to 6 μm) increasing meltability and elasticity in Camembert, potentially due to the greater integration of fat within the network (Michalski et al. 2003).

These studies illustrate how microscopy can be used to understand the microstructure of the native building blocks of cheese, the modification of this structure, and the formation of new microstructures within intermediate samples and final products. The practical application of microscopy in process characterization and optimization and useful problem solving is explored in further detail in the following sections.

4.3. Understanding and Benchmarking Cheese Manufacturing

Microscopy allows manufacturers to understand how product microstructure develops within an existing manufacturing process. An example is given in **Figure 4**, where the structures at key intermediate stages within the manufacture of Cheddar cheese are shown. This approach, which can be conducted at the laboratory or process scale, illustrates the effect of individual unit operations. Although the steps involved in the manufacture of Cheddar cheese are extensively documented, microscopy can provide valuable new insights into this process under conditions specifically relevant to a specific process or individual manufacturer. A complete evaluation of such a manufacturing process can provide a benchmark for comparison in future optimization studies.

Quantitative measures that can aid benchmarking include (*a*) sample porosity, (*b*) the size distribution of fat globules or nonglobular free fat (the fat that is not protected by the MFGM and susceptible to oiling off), (*c*) the volume fraction of fat or protein, (*d*) the size of curd junctions, and (*e*) other structural features not limited to fat and protein (see Section 3.2). Microscopy can also be used to compare microstructure and process changes obtained at the laboratory, pilot, and process scales to illustrate the impact of scaling, although there are few examples at the commercial scale in the literature. A key challenge at any scale is the small fraction of product sampled in each batch. This can be overcome by the use of replicate samples that are drawn

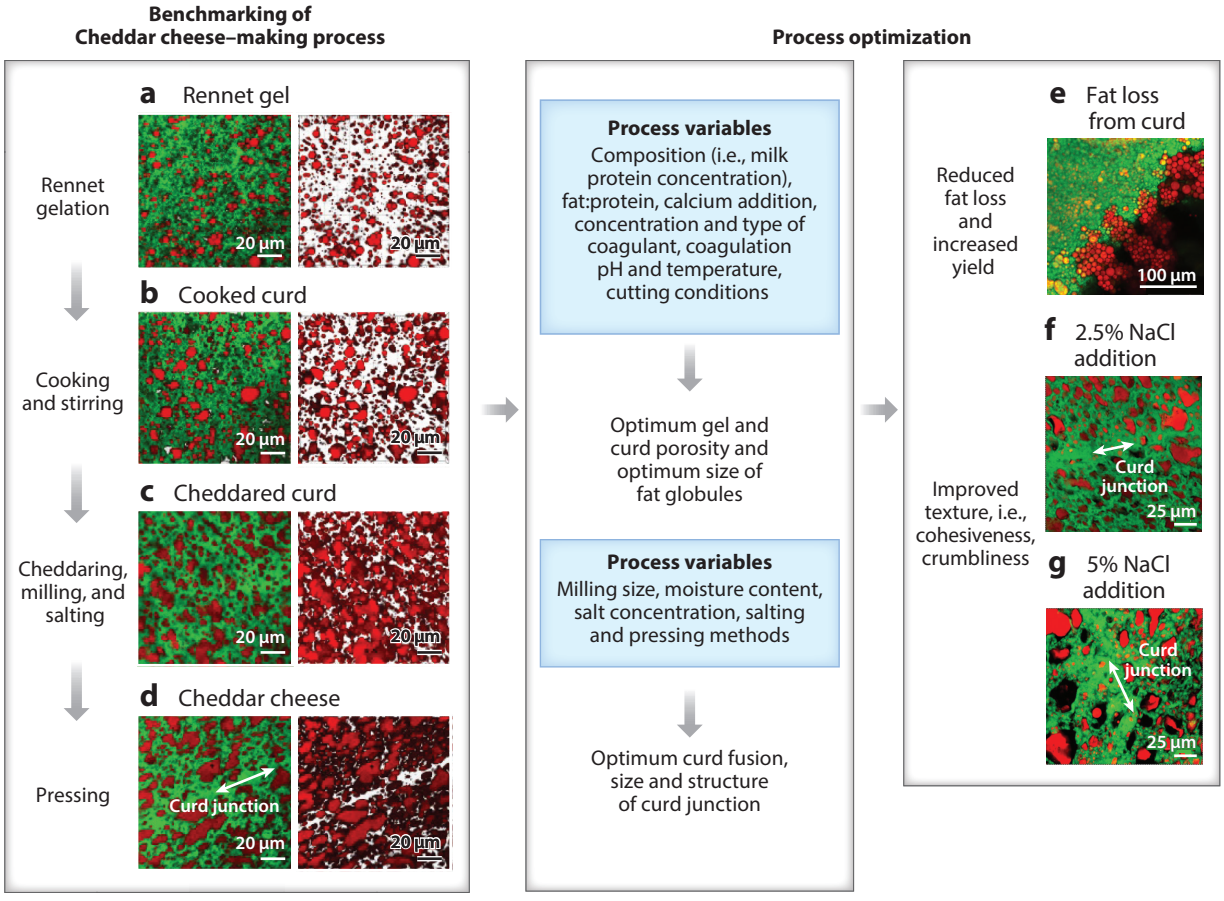


Figure 4

The application of microscopy to benchmark and subsequently optimize a manufacturing process. Left: Benchmarking a Cheddar cheese–making process; samples are collected after (*a*) rennet gelation, (*b*) cooking and stirring of the curd, (*c*) Cheddaring, milling, and salting, and (*d*) pressing. Each microstructural image in panels *a–d* shows the overlay of fat and protein and the microstructure of fat alone. Center and right: The standard benchmarked process can be optimized to reduce fat loss, increase yield, and optimize texture. Examples of optimization include (*e*) a reduction of fat loss and increase in yield, (*f*) the optimization of curd junctions within cheese with 2.5% NaCl addition, resulting in fine curd junctions, and (*g*) 5% NaCl addition resulting in coarse curd junctions. Fat appears red and protein appears green in these images. Images in panels *a–d* and *f–g* adapted with permission from Ong et al. (2017, 2020a). Image in panel *e* is an unpublished image from L. Ong and A. Aldalur.

from each stage of the process and careful selection of the sampling region used for microscopy analysis (e.g., surface versus geometric center of a curd granule). Although differences between production runs are typically small (To et al. 2020), sampling from multiple production runs can also increase confidence in microstructural data.

Collection of qualitative and quantitative microstructural data for a process enables optimization at key process stages, for example, using the process variables identified in **Figure 4**. Correlations between microstructural features and changes in fat loss or product properties, such as texture, can assist in increasing yield or optimizing the texture.

Benchmarking provides a snapshot and point of reference when subsequent process changes are made. These could include changes in

- composition and ingredient formulation, e.g., seasonal variation, substitution, or customer requirements.
- equipment, e.g., during replacement or upgrade.
- process variables (**Figure 4**) or control procedures.

A systematic evaluation of these changes allows optimization of key unit operations (Ong et al. 2017), as described in the next section.

4.4. Process Optimization

Microscopy can help manufacturers optimize processes in which the key process steps are benchmarked, as shown in **Figure 4**, acting as a tool complementary to process engineering steps taken to increase yield, reduce waste, and improve process control. Individual formulations, process steps, or unit operations can also be examined in isolation. Examples explored here include the optimization of milk protein concentration; the optimization of processing steps, including curd cutting, dry salting, and stretching; and the optimization of storage or ripening processes following production. A more extensive list of process variables that can be optimized is outlined in **Figure 4*b***. Process optimization can also be used to identify critical control points, where there is a strong link between process variables and product quality, where process control should be implemented.

Milk protein concentration, which influences protein retention and impacts profitability, can be tracked and optimized using microscopy. Protein is typically standardized to ∼4% w/w protein to minimize variation due to season, feed, lactation, or breed (Chen et al. 2014). Low concentration factor UF retentate can also be used to increase protein content, and ∼5% w/w (total protein) has been used in Cheddar or pizza cheese to increase yield (Govindasamy-Lucey et al. 2005, Ong et al. 2013, Panthi et al. 2019). 3D CLSM microscopy and quantitative image analysis have shown that 5% w/w milk protein provides an optimum gel porosity, reducing fat and protein loss to the whey (Ong et al. 2013). Microscopy has also shown that addition of calcium chloride decreased gel porosity and lowered fat loss to the whey for Cheddar cheese (Ong et al. 2015).

Microscopy can provide insights into process steps, including curd cutting and cooking, to reduce fat loss and increase yield (**Figure 4*e***). In sheep's milk cheese, for example, digital images of curd grain size were complemented by CLSM and cryo-SEM images that provided quantitative data on porosity as well as the size and shape of fat droplets. These data were then correlated with fat loss and physical properties to show that small curd grains (∼12–15 mm^2 versus 45–53 mm^2) led to higher fat loss, and higher cooking temperatures (45°C versus 36°C) increased the number of nonglobular fat droplets, reducing porosity and increasing hardness (Aldalur et al. 2019).

Studies of cheese microstructure have also shown the importance of controlling dry salting in Cheddar cheese production, as highly concentrated salt pockets that occur due to uneven salt distribution result in coarse and thick curd junctions (**Figure 4*g*** versus **4*f***) (Ong et al. 2020a). The thickness of the curd junctions and size of fat globules could be quantified by image analysis, showing how exposure to high concentrations of NaCl increased protein hydration at the curd surface and caused the rupture of fat globules, leading to fat loss during mixing and pressing and a less cohesive cheese. The optimal salt concentration for brine salting Ragusano cheese has also been determined by examining cheese porosity, as high salt concentrations can lead to the formation of a surface barrier layer (Melilli et al. 2005).

Further cheese properties, such as melting, may be optimized using insights into the microstructure of Mozzarella cheese and the effect of mechanical and thermal treatment during cooking and stretching of curd. For example, a higher stretching speed and temperature leads to

an increase in the circularity and Feret's diameter of fat globules. Large fat globules then positively influence the meltability and free oil of cheese but negatively influence stretching properties (Ma et al. 2013).

The storage or ripening conditions can further alter cheese microstructure and affect cheese properties. Microscopy analysis is complementary to chemical analysis and has been widely applied to monitor structural degradation during storage and in the supply chain. Structural changes during accelerated Cheddar cheese ripening, for example, could be observed by CLSM. Quantitative image analysis indicated fewer vertices in the protein network and less protein branching at 20°C compared to 8°C due to increased proteolysis (Soodam et al. 2017).

Freezing has been widely investigated, as it can contribute to a more flexible supply chain, provide opportunities to preserve stock, and allow manufacturers to reach more distant markets (Alinovi et al. 2020a,b; To et al. 2020). Ice crystals can have a negative impact, however, disrupting the casein matrix and altering the product microstructure (Kuo & Gunasekaran 2003, 2009). Hyperspectral imaging, FTIR, and Raman spectroscopy have been increasingly used to distinguish between fresh and freeze-thawed food samples, including mushrooms, fish, and meat (Dalvi-Isfahan et al. 2019), although there are fewer studies on cheese. CLSM and confocal Raman have been used to map large water domains formed during the freezing and thawing of high-moisture cream cheese (Alinovi et al. 2020b), although Raman spectroscopy indicated no differences between the chilled and freeze-thawed samples. Further development of these techniques is required to provide a quantitative assessment of the changes in protein or fat structures during freezing and the role of sugars and stabilizers in the stability of cream cheese.

Microscopy can also be used to identify and understand critical control points within a cheese-making process that impact product quality. One example is cream cheese production. The development of cheese microstructure during a benchmarked process is illustrated in **Figure 5*a***. The initial structuring of the building blocks by homogenization results in fat and protein clusters of ~1 μm in size (Ong et al. 2018) that aggregate during fermentation. pH is a critical parameter and gel formation is a critical control point in this process. pH impacts on gel porosity and affects the structure and properties of subsequent samples, including the gel particles collected after heating, the cheese curd collected after whey separation, and the final cream cheese product (**Figure 5*a***) (Ong et al. 2020b). The control of pH to reduce or eliminate variation is therefore desirable. At pH 5, corpuscular aggregates of protein and fat appear to swell, leading to a softer cheese (**Figure 5*b***), with synchrotron-FTIR microspectroscopy indicating a larger concentration of β-turn structure within the protein network. At pH 4.5, the cheese microstructure is denser and a higher concentration of aggregated β-sheet structure contributes to an increase in cheese hardness (**Figure 5*b***). An understanding of this benchmarked process and the structural variation that occurs with changes in process variables (in this case, pH) allows manufacturers to implement process control steps to minimize variation in texture and potentially tailor product properties.

4.5. Reverse Engineering

Reverse engineering is a method of working backward from a known product microstructure, generating a formulation and production process that can recreate this target structure and its associated properties. Reverse engineering typically involves a finished product, including competitor products, but can also be applied to intermediate samples within a process. Given the proprietary nature of these activities, they are not typically described in the literature. There is a good example of reverse engineering, however, applied to a scaled-down stirred yogurt process, which was designed to match a reference product produced at a pilot scale (Moussier et al. 2019). The same principles can also be applied to reverse engineer milk products from different species or plant-based milk, resulting in products that resemble typical dairy products.

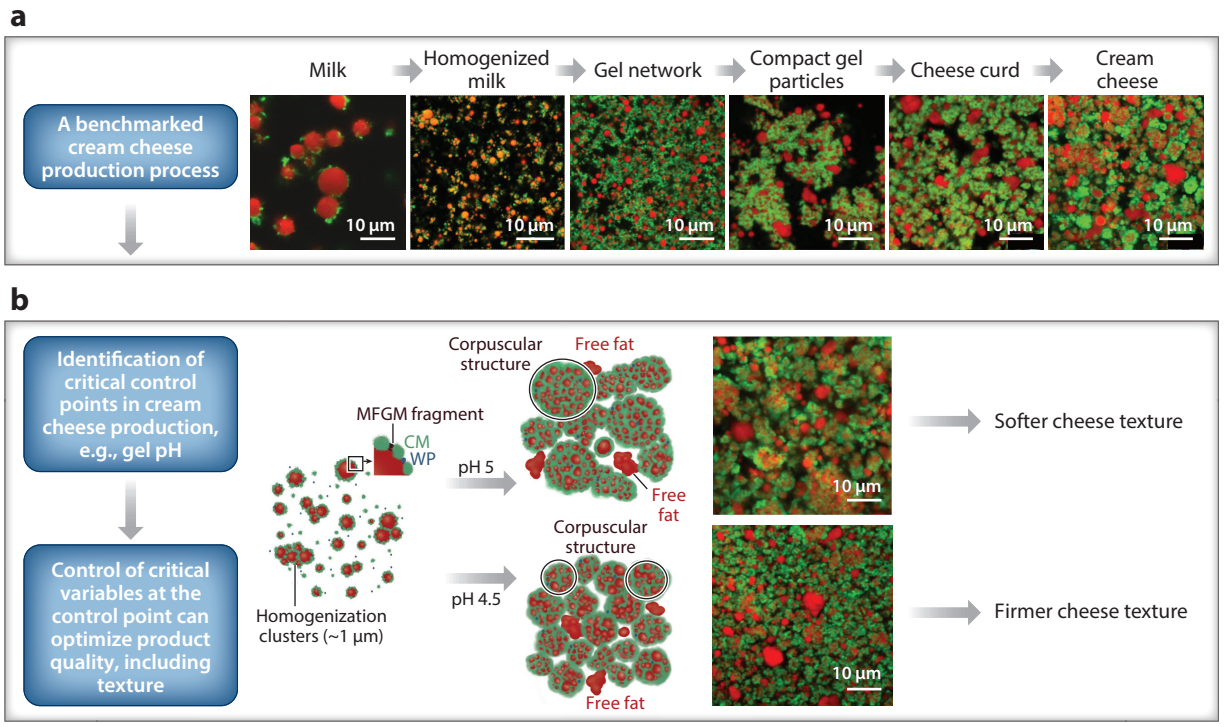


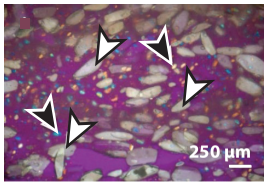
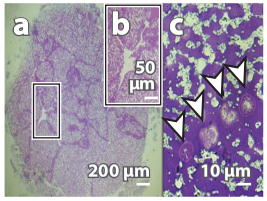
Figure 5

The application of microscopy to identify critical control points and critical control variables in a manufacturing process. (*a*) A benchmarked cream cheese production process. (*b*) The identification of a critical control point at the point of gel formation in cream cheese manufacture, where the alteration of gel pH (pH 5 versus pH 4.5) leads to a change in product quality, including cheese microstructure and texture. Fat appears red and protein appears green in these images. Images adapted with permission from Ong et al. (2018, 2020b). Abbreviations: CM, casein micelle; MFGM, milk fat globule membrane; WP, whey protein.

One example is the modification of buffalo milk properties to produce dairy products with properties like those made from bovine milk. Buffalo milk has twice the fat content of bovine milk (~7.4% w/w versus ~4% w/w, respectively) and fat globules are on average around 50% larger than in bovine milk (~5.0 µm versus ~3.3 µm, respectively) (Nguyen et al. 2015a, Ong et al. 2011). Knowledge of this microstructure allows the reverse engineering of milk properties, e.g., by homogenization, to match the size of homogenized fat globules in bovine milk, preventing syneresis and the formation of free fat (Nguyen et al. 2015b). The high protein content in buffalo milk is favored, however, for high moisture Mozzarella cheese, as it contributes to a microstructure with increased protein alignment that stretches better (Nguyen et al. 2017b).

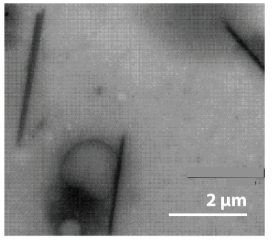
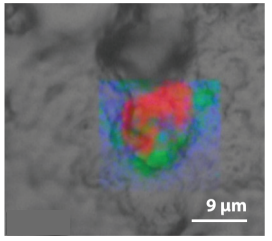
New plant-based products face several challenges that can be assisted by reverse engineering. Most cheese alternatives currently available on the market are low in protein and feature starches and stabilizers (Nicolás Saraco & Blaxland 2020). Key problems include texture and functionality (Mattice & Marangoni 2020), and research is needed to match the functionality of bovine casein using plant-based proteins for cheese development. A fundamental understanding of the microstructure of the plant-based building blocks, the different factors that affect the initial gel assembly, and the microstructure of the final product will assist this development (Kyriakopoulou et al. 2021, Marinea et al. 2021). This approach was demonstrated in a recent study that developed almond gels targeting a microstructure similar to the protein network of bovine acid gels, where

Table 2 The application of microscopic techniques to identify and characterize cheese defects

Cheese defect	Cheese type	Microscopy techniques	Key findings	Industrial significance	Representative image[a]	Image description
Grittiness from surface ikaite and struvite crystals	Soft, washed-rind cheese	PLM	Perceived grittiness was associated with growth of ikaite and struvite crystals that developed due to the alkaline surface conditions during ripening (Polowsky et al. 2018a,b)	Allows nonoptimal conditions for ripening and crystal formation to be identified and crystal characteristics to be identified	 (Polowsky et al. 2018a)	Polarized light micrographs of crystals isolated from cheese smear material. Crystals of ikaite (*white arrows*) and struvite (*black arrows*) are shown. Scale bar is 250 µm (Polowsky et al. 2018a)
Surface white spot	Hard and extrahard cheese	LM, TEM	Growth of surface spots was associated with incomplete aggregation of curd granules (D'Incecco et al. 2016)	Surface defects to be avoided by improving syneresis through fine temperature control	 (D'Incecco et al. 2016)	Light microscopy of semithin section (2–5 µm) of a surface spot stained with toluidine blue. (*a*) The curd junctions are visible as darker lines. Scale bar is 200 µm. (*b*) Detail of the large hole in which junctions converge. Scale bar is 50 µm. (*c*) Microcrystals (*arrows*) along a curd junction. Scale bar is 10 µm (D'Incecco et al. 2016)

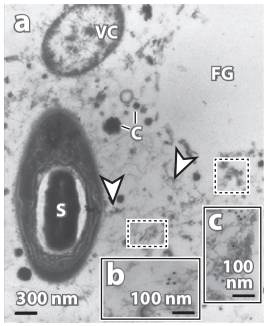
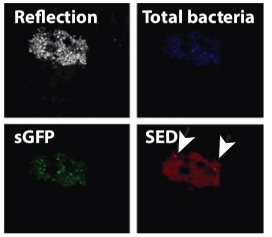
(*Continued*)

Table 2 (*Continued*)

Cheese defect	Cheese type	Microscopy techniques	Key findings	Industrial significance	Representative image[a]	Image description
Black spot	Cheddar	TEM	TEM revealed hair-like nanorod structures within black spots, suggesting possible contamination of milk with bismuth subnitrate from intramammary teat sealant (Lay et al. 2007)	Procedural issues with intramammary teat sealant may be identified	 (Lay et al. 2007)	TEM image of typical hair-like nanorod structures found within black spot defects (Lay et al. 2007)
Pink discoloration	Ripened cheese	RISE microscopy	RISE microscopy revealed the chemical distribution of carotenoid (lycopane), fat, and protein in the cheese matrix, which was complementary to metagenomic sequencing used to identify the presence of *Thermus* organisms and the genes responsible for carotenoid production (Quigley et al. 2016) A recent work reported *Serratia liquefaciens* could also cause the pink discoloration in Pecorino Toscano cheese (Martelli et al. 2020)	May assist in the elimination of pink discoloration defects. The novel method could also be used more widely to investigate food defects caused by microbes of unknown origin	 (Quigley et al. 2016)	Overlay of intensity images of the cheese matrix (*gray*) and maps of the chemical composition obtained from RISE microscopy analysis of the pink discoloration region: red, carotenoid (lycopane); blue, proteins; green, lipids (Quigley et al. 2016)

(*Continued*)

Table 2 (*Continued*)

Cheese defect	Cheese type	Microscopy techniques	Key findings	Industrial significance	Representative image[a]	Image description
Late blowing defect	Hard cheese	TEM	Immunogold labeling and TEM indicated immunoglobulin A and, in some cases, immunoglobulin M were involved in clustering of *Clostridium* spores or vegetative cells and adhesion to fat globules (D'Incecco & Pellegrino 2018; D'Incecco et al. 2015, 2018b)	Late blowing defects may be lessened by the development of desporification methods	 (D'Incecco & Pellegrino 2018)	(*a*) TEM image of an immunolabelled ultrathin section of a spore (S) and vegetative cell (VC) interacting with a fat globule (FG) via amorphous material (*arrows*). Scale bar is 300 nm. Gold-labeled IgA antibodies (black dots) on the amorphous material within the dashed lines are enlarged in panels *b* and *c*. C, casein micelle. Scale bars in panels *b* and *c* are 100 nm (D'Incecco & Pellegrino 2018)
Contamination from pathogenic bacteria	Semihard cheese	CLSM	CLSM could discriminate doped sGFP expressing *Staphylococcus aureus* from cheese starter bacteria and combined with immunochemistry could also detect the presence of SED (Fleurot et al. 2014)	Can be used to track the distribution of specific doped bacteria and monitor gene expression, e.g., toxin production associated with food contamination	 (Fleurot et al. 2014)	CLSM image of *S. aureus* on the cheese surface at day 15. The structure of the cheese sample is visualized by the reflection of the 405-nm laser diode in a grayscale image. Dairy bacteria and pathogens are blue stained with DAPI, *sgfp* expressing *S. aureus* strains are green and SED is red. SED-positive bacteria are indicated by arrows (Fleurot et al. 2014)

[a]The images used in this table were reprinted from cited references, with permission from corresponding publisher.

Abbreviations: CLSM, confocal laser scanning microscopy; DAPI, 4′,6-diamino-2-phenylindole; LM, light microscopy; PLM, polarized light microscopy; RISE, Raman integrated scanning electron; SED, staphylococcal enterotoxin D; sGFP, superfolder green fluorescent protein; TEM, transmission election microscopy.

fat is embedded in the network (Devnani et al. 2020), although this study did not create a cheese analog.

Reverse engineering can also be used to assist the development of 3D-printed food. Techniques such as SEM, microcomputed tomography (μCT), and machine learning were used to obtain a desired 3D geometry for a composite product on printing to match desired mechanical properties (Yanamandra et al. 2020). In addition, there is potential to extend the process of reverse engineering to optimize food processing at a systems level, as described recently in a review on multicriteria reverse engineering that outlined numerical and computational approaches that can be taken to optimize competing factors affecting product quality (Thomopoulos et al. 2019).

4.6. Quality Control and Troubleshooting Production Problems

Microstructure analysis is also useful for troubleshooting production problems and can help manufacturers to understand the cause of various surface or internal defects that occur during production, ripening, or storage. These defects include crystals, surface spots, or discoloration of several colors, arising from physical processes, chemical or microbial contamination, and late blowing defects (**Table 2**).

In many cases, microscopy has been used to identify and characterize the cause of the defect as well as to localize these defects relative to other cheese components such as protein and fat, which may affect defect formation and/or subsequent development. Microscopy has also been used to better understand well-known problems, such as gas blowing defects and the possible role of immunoglobulins in spore adhesion to fat globules. Most of these studies are qualitative, although these methods could be extended to quantify defects such as crystals (Polowsky et al. 2018a). Other techniques have promise for crystal visualization (e.g., second harmonic generation imaging), but the identification may require complementary techniques such as X-ray diffraction or energy-dispersive X-ray spectroscopy to confirm the chemical composition (Burdikova et al. 2015a). Additional information can also be obtained by combining microscopy with microbial identification, quantification, or DNA sequencing. For example, the doping of fluorescent food pathogens can also differentiate pathogenic bacteria from dairy starter cultures; when combined with immunohistochemistry, these techniques can also be used to monitor gene expression, including enterotoxin production (Fleurot et al. 2014). The combination of microscopy with techniques such as next-generation sequencing or whole-genome sequencing (Jagadeesan et al. 2019, Yeluri Jonnala et al. 2018) also has the potential to help cheese producers better understand microbial diversity and quickly identify spoilage organisms to solve quality problems and maintain quality control.

SUMMARY POINTS

1. Microscopy has great potential as a useful tool for dairy product manufacture, increasing the understanding of manufacturing processes and assisting process optimization. These insights are related and complementary to the link between cheese microstructure and product quality and functionality.
2. Although an individual microscopy technique may not be able to investigate the microstructure of dairy samples at all length scales relevant to manufacturing, a combination of different microscopy tools, together with quantitative image analysis, can help establish correlations between key structural traits and processing steps or variables. Recent advances, including label-free techniques and the integration of other analytical

techniques, have allowed for more information to be obtained from images, including chemical maps and mechanical properties.

3. Intermediate samples, assessed throughout the manufacturing process, allow manufacturers to benchmark and optimize existing processes, increasing yield and reducing by-products. Microscopy analysis can also be used to identify critical control points, assisting manufacturers in minimizing variation in product quality and tailoring new product textures.
4. Knowledge of a desired product microstructure can also be used to reverse engineer new processes to obtain key product structural and functional properties, although there are few examples of this commercially focused activity in the literature.
5. Microscopy can assist in the identification and characterization of defects detected during production, ripening, or storage. When combined with chemical or microbial analysis or DNA sequencing, microscopy can also help clarify more complex defects, including microbially induced defects, and solve quality problems.

FUTURE ISSUES

1. The field of food or cheese microstructure analysis benefits from advances in biomedical imaging, but many of these techniques require adaptation. Sample preparation techniques need to be developed specifically for food samples to ensure samples remain hydrated and the microstructure is preserved during imaging, avoiding fixing, staining, drying, and sectioning of thin samples, which can cause artefacts. The rapid development of new biological techniques will provide significant opportunities for the field if these challenges can be addressed.
2. The intersection between cheese microscopy and other fields is likely to provide new insights. Intersections with microbial analysis, metabolomics, proteomics, lipidomics, and transcriptomics will provide new information complementary to images. Techniques from computer science and engineering will enable new methods for the analysis and control of data, processes, and systems. Additive manufacturing and reverse engineering also offer the promise of new routes to product manufacture.
3. Advances in microscopy technologies and image analysis software will allow faster acquisition and image quantification. The large sample data sets generated also hold potential for the application of machine learning or deep learning techniques for the prediction of product functionality and the optimization of both processes and product quality attributes.
4. The microscopic analysis of dairy samples during manufacturing typically requires at-line, or more commonly offline, analysis by experts. Techniques for faster and higher throughput assessment of intermediate or product structure may see broader application within factories.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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